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ORIGINAL RESEARCH ARTICLE

Slow Sand Filters as a part of integrated protection of seedlings against disease in forest nurseries

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Abstract. Slow Sand Filters (SSF) are a biological method used to protect nursery plants, from pathogen infections which can cause serious diseases in many forest tree species. Thanks to SSF application the number of phytopathogens in nurseries can be significantly reduced, as demonstrated by many field and greenhouse experiments (e.g. in Polish nurseries, and for horticultural crops in Germany and The Netherlands).

In this study, the effect of pollution from fertilizers and fungicides used in agriculture (e.g. PCNB) on the efficiency of SSFs was assessed. A quantitative analysis was performed of the copiotrophic and oligotrophic bacterial composition colonizing SSF biofilms. The efficiency with which selected Oomycete strains belonging to the genus *Phytophthora* (*P. alni, P. cactorum, P. plurivora*) were removed from water was determined based on genetic material (DNA of the organisms) found in the SSF filtrate. Specific primers and TaqMan probes (qPCR) appeared to be the most sensitive molecular methods. Moreover, the microbiological analysis of SSF biofilm performed with selective media allowed the growth of copiothrophic and oligothrophic bacteria to be estimated. The influence of fungicide (PCNB) and N-fertilizer on the number of bacteria in each biofilm was also evaluated.

The pollution of water with fertilizer (being used for plant irrigation) was demonstrated to reduce the efficiency of filtration more than fungicide addition (the amount of DNA from those investigated pathogens in the water decreased with time). The amount of bacteria in SSF biofilm readily increased after application of N-fertilizer in contrast to fungicide (PCNB) addition.

Key words: Phytopathogen, biofilm, SSF, PCNB, mineral fertilizer

1. Introduction

Taking up water from natural surface water intakes (waterways) and its use for watering plants in nurseries, is risky because of spreading phytopathogens and the development of many plant diseases (Runia 1995; Wohanka 1995; Ehret et al., 2001). Water from nearby lakes and ponds is most often used for irrigation of nurseries. Frequently, these shots are located in the catchment area of the land used for agriculture, and that is why they are mostly contaminated with phytopathogens and products originating from agricultural sources (fertilizers, pesticides). Improper or excessive use of chemical plant protection products involves numerous risks, including contamination of the environment (residue of plant protection products), reduction of biodiversity and occurrence of resistant organisms, harmful to plants.

Slow sand filters with biological membrane (biofilm) are designed to eliminate pathogens from water (Kubiak, Oszako 2011), among others, oomycetes belonging to the genus *Phytophthora*, which affect plants for plantings. Phytopathogens removal efficiency through filters depends on their types and activity of microbial processes occurring in their biofilters (Davey and O'toole 2000), which are also affected by anthropogenic pollution (e.g., caused by agricultural activities).

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The aim of the study was to assess the impact of fertilizer (N) and fungicide (PCNB) on the effectiveness of working of slow sand filters (SSF) in the removal of phytopathogens from water used for plants watering. The analysis of the impact of water pollution (e.g., fertilizer), for quantitative composition of bacteria colonizing the biofilms of filters being used for watering plants in nurseries, was carried out too.

2. Materials and Methods

Analysis of the effectiveness of the elimination of phytopathogens by biofilters (SSF)

Was prepared in three variants. A) Biofilter 1 – control (natural); B) biofilter 2 – enriched with mineral fertilizer (in the amount of 55 ml/40 l of water with the composition as follows: total nitrogen – 3%, nitrate nitrogen – 1%, urea nitrogen – 2%, phosphorus pentoxide – 5%, potassium oxide – 7%, boron – 0.02%, copper – 0.008, iron – 0.03%, manganese – 0.015%, molybdate – 0.002%, zinc – 0.015%); C) biofilter 3 – containing additive of 1 g fungicide (PCNB) dissolved in 50 ml of 96% ethanol (fig. 1). Pure cultures of *Phytophthora alni, P. plurivora, and P. cactorum* were added to the container where 120 l of tap water was accumulated. Isolates that originated from the collection of Forest Research Institute (IBL) were incubated for 4 weeks in the dark at room temperature, growing on the liquid vegetable medium (V8). The day before the filtration had started, the above cultures were transferred for 24 hours to the temperature of 4°C (to initiate spores production), and then were homogenized and mixed together in equal volumes. After adding the mixture of oomycetes to tap water, the filtration system worked for two days without any break. The water was pumped out in parallel to the three filters (SSF) having different biofilm variants: filter 1 (F1) – biofilm control, filter 2 (F2) – a biofilm with a mineral fertilizer, and filter 3 (F3) – biofilm with PCNB. Samples of water were collected prior to and during the filtration at specified intervals (3, 6, 9, 12, 24, and 48 hours after the filtration had started). Water samples were collected into sterile flasks (1 l of volume) from water storage tank (to determine the amount of pathogen DNA prior to filtration), and directly from filtrates (to compare the amount of pathogen DNA after filtration). The isolation of genomic DNA, and quantitative analysis of selected oomycetes species by qPCR (real-time PCR) allowed us to evaluate the presence of pathogen DNA in both unfiltered water and filtrates.

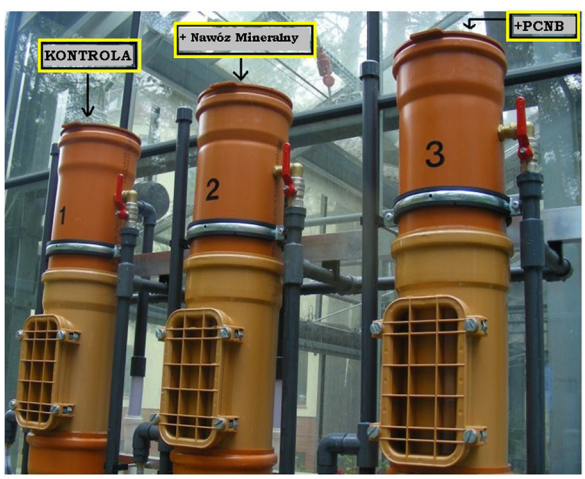

Photo 1. Prototypes of Slow Send Filters installed in Forest Research Institute greenhouse

Preparation of water samples for analysis of molecular qPCR

Water samples were filtered twice with a vacuum pump by a Millipore membrane filter (47 mm Ø) (Millipore ®) with the pore diameter of 11 microns; the filtrate was further filtered through a membrane filter with a pore diameter of 5 mm in order to retain the spores and mycelia fragments of oomycetes. Then, the membrane filter with the sludge was placed in Eppendorf tubes to which 2 ml of sterile and distilled water was added, and the water was shaken for 60 min at room temperature at a speed of 1400 rpm. The suspension thus obtained was centrifuged for 15 min at 13 000 rpm; the filter was removed and the precipitate was collected at the bottom and treated as a starting material. DNA isolation was performed using a set of GenElute PLANT GENOMIC DNA Miniprep Kit from Sigma Aldrich according to the manufacturer's instructions with some modifications. Namely, during the cell lysis, 20 µl of glass beads was added to Eppendorf tubes containing the precipitate of water and shaken with lysis buffer for 15 min at 65°C (1400 rpm), and then for 15 min at room temperature under manual MoBio vortexing at maximum speed; the action was repeated twice. Isolated genomic DNA was purified by Clean Up kit A & A Biotechnology and separated by electrophoresis in 1% agarose gel.

Quantitative analysis – qPCR

In order to determine the presence and to estimate the DNA quantity of selected oomycetes species (before and after filtration), both molecular primers and TaqMan probes were used (tab. 1). Each sample was analyzed

Table 1. Sequences of molecular primers and probes TaqMan types complementary to the DNA of selected phytopathogens used in quantitative analysis – qPCR

Species	Sequences of starters	Probe of TaqMan type
Phytophtora alni	CTGTCGATGTCAAAGTTG ATGGGTTTAAAAGATAAGGG	[HEX]ACCCAAACGCTCGCCATGATA[HBQ1]
Phytophtora cactorum:	ACGTGAACCGTTTCAAAC CAGCCGCCAACAATAAAG	[TET]CAGCCGCCACCAGACAAGAC[HBQ1]
Phytophtora plurivora	CCGTATCAACCCTTTTAG GCAGTATAATCAGTATTGTAGA	[6FAM]CCCAGACCGAAGTCCAAACAT[HBQ1]

three times, and therefore the result is the average of these three replicates. The composition of the qPCR reaction mixture to a volume of 20 µl was as follows: 10 µl of mixture of Lumino Ct, 2 µl primer mixture (10 mM each), 2 µl probe (*P. alni* – 5 mM, *P. cactorum* – 10 mM, *P . plurivora* – 1 mM), 2 µl of DNA (approximately 20 ng), 4 µl of MilliQ-water. Quantitative PCR reaction consisted of the following cycles: denaturation initial – 94°C for 3 min, amplification – 40 cycles, denaturation – 94°C for 30 s, primer attachment of – 55°C for 30 s, elongation of the primer – 72°C for 30 s. The obtained quantitative results were statistically analyzed. The differences between the mean values of respondents reduction of pathogens in all variants of biofilms in filters F1, F2, and F3 were tested using multivariate ANOVA in STATISTICA v.8.0, assuming $\alpha = 0.05$.

Analysis of the quantitative composition of the biofilm colonizing bacteria SSF

In order to assess the presence of oligotrophic and copiotrofic bacteria in the biofilm, the microbial analysis was performed by dilution and culture on solid media. 1 g of biofilm from each variant of SSF biofilter was taken and transferred to 9 ml of sterile 0.85% saline, and then the whole product was shaken for 15 min in a bath. In the succeeding stage, a further series of dilution of the parent sample in the tubes containing 9 ml of sterile saline solution was done, transferring 1 ml of the previous dilution to the next one. Then, inoculation by bacteria was done using 0.1 ml of the suspension on nutrient agar (for copiotrofic bacteria) or nutrient agar diluted 1000-fold (for oligotrophic bacteria) from a dilution series (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) in triplicates. After the incubation process, which was done at room temperature for 3 days (copiotrofs) and 7 days (oligotrofs) the number of grown colonies was counted. Statistical differences between mean values for copiotrofic bacteria (copio) and oligotrophic (oligo) in all variants of biofilms (in filters F1, F2, and F3) were tested using univariate ANOVA in STATISTICA v.8.0.

3. Results

Quantitative results obtained using qPCR with TaqMan probes provided data on the basis of which the degree of elimination of pathogens was expressed as reduced content of their DNA in the filtered water. DNA reduction of *P. alni* in the control biofilter and biofilter with PCBN proceeded smoothly and efficiently, namely the quantity of DNA was decreasing with increasing time of filtration, and reached the high level of DNA reduction (99.9% and 99.1% respectively) after 48 hour of filtration. However, in the biofilter with fertilizer, the elimination *P. alni* was significantly affected. Till 6th hour of continuous filtration, the DNA reduction of *P. alni* was recorded at the level of 72.5%, and subsequent measurements after 9 and 12 hours showed changes in the effectiveness of the reduction to only 43.5%, then after 24 and 48 hours the filtration efficiency again increased by almost half, and reached after 48 hours, the level of 83.7% (fig. 1).

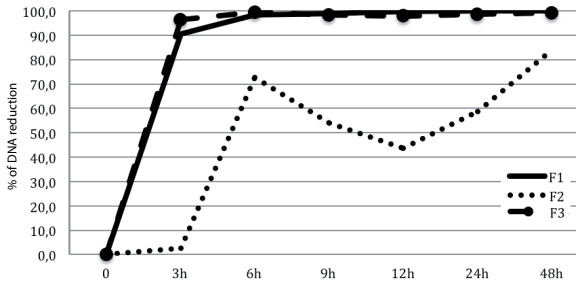

Figure 1. Reducing the amount of DNA of *P. alni* (in%) in three variants of SSF biofilter during 48 hours of water filtration inoculated with phytopathogens

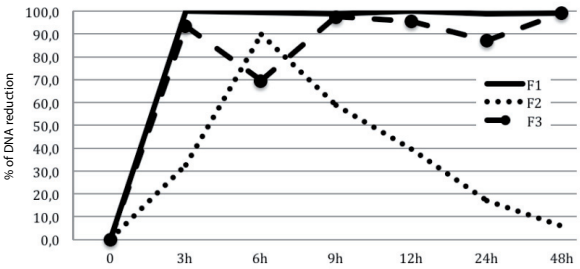

Figure 2. Reduction of *P. cactorum* DNA (in %) in three variants of SSF biofilters during 48 hours of water filtration inoculated with phytopathogens

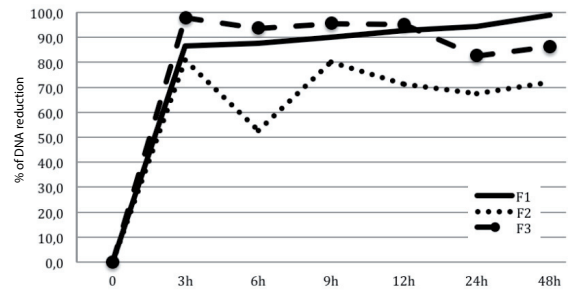

Figure 3. Reducing the amount of *P. plurivora* DNA (in%) in 3 variants of SSF biofilters during 48 hours of water filtration inoculated with phytopathogens

Reducing the amount of *P. cactorum* DNA in the control biofilter proceeded efficiently and evenly and reached the level of 99.1% reduction of pathogen DNA after 48 hours of filtration. The biofilter with PCBN after two days of filtration, to reduce the level of DNA, was also high at 99%. However, after 6 and 24 hours, there was a slight decrease in filtration efficiency for the level of 69.4% and 86.9%, respectively. In the biofilter with fertilizer, the elimination of *P. cactorum* was high (at 89.7%) until the expiry of six hours, and then showed a tendency to decrease, reaching the value only 6% after 48 hours (fig. 2).

Reducing the amount of *P. plurivora* DNA in the control biofilter proceeded efficiently and evenly

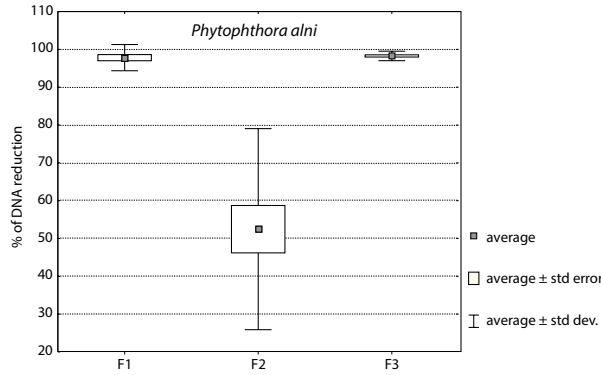

Figure 4. The reduction of pathogen P. alni (Pa) by the application of three variants of filters: F1 (control), F2 (phosphite), and F3 (PCNB). Differences between the reduction of P. alni and the type of filter are statistically significant ($p = 0.00000$).

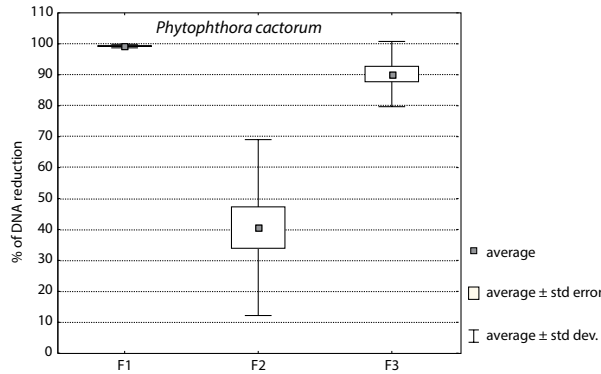

Figure 5. The reduction of pathogen *P. cactorum* (Pc) in three variants of filters: F1 (control), F2 (phosphite), and F3 (PCNB). Differences between the reduction of *P. cactorum* and the type of filter are statistically significant ($p = 0.00000$).

reaching the level of 99% reduction of pathogen DNA after 48 hours of filtration. During filtration (in the biofilter with PCBN) a low decrease of filtration efficiency was observed after 3 and 12 hours (the levels of 93.9% and 82.8%, respectively) and after 48 hours the performance of pathogen elimination from contaminated water was high again, 86.5%. In the biofilter with mineral fertilizer, the filtration efficiency was interfered after 3, 9, and 24 hours, when the reduction of *P. plurivora* DNA amounted to 52.9%,

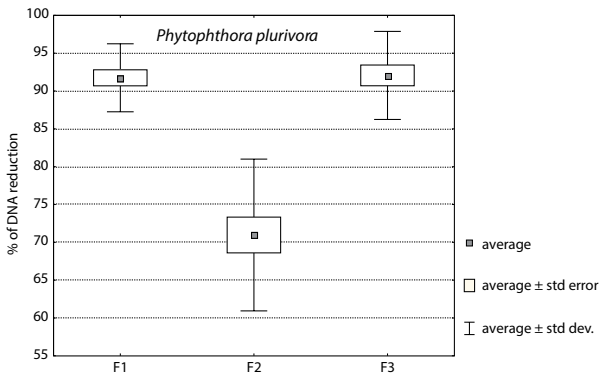

Figure 6. The reduction of pathogen *P. plurivora* (Pp) in three variants of filters: F1 (control), F2 (phosphite), and F3 (PCNB). Differences between the reduction of *P. plurivora* and the kind of filter are statistically significant ($p = 0.00000$).

71.4%, and 67.7%, respectively; but after 48 hours of filtration it rose again to 72.3% (fig. 3).

Based on the results of the statistical analysis it was found that the effectiveness of the biofilm in the filter depends on the measurement time and the type of filter contamination of agricultural origin. After the addition of a mineral fertilizer (F2), a statistically significant reduction in the filtration efficiency of the tested species was shown, regardless of the pathogen (*P. alni, P. cactorum* or *P. plurivora*), for $\alpha = 0.05$ (fig. 4, 5, and 6). When PCNB was added to the filter (F3), there was no statistically significant difference with the control filter (F1), calculated using the Tuckey test for heterogeneous samples, $\alpha = 0.05$. The effectiveness of the elimination of 3 studied pathogenic species from contaminated water stabilized and reached its maximum after 48 hours (fig. 7).

Quantitative analysis of the biofilm collected from the control filter No. 1 showed that the number of oligotrophic and copiotrophic bacteria was similar and amounted to 23×10^6 and 21×10^6 JTK / 1 g of biofilm (colony forming units in 1 g of biofilm). The biofilm samples collected from filter No. 2 after application of fertilizer revealed 77% more copiotrophs and 33% oligotrophs (compared to control); their number was: 40.8×10^6 and 28×10^6. In biofilm samples after application of PCNB, 18% less copiotrophs and 17% less oligotrophs were noted (compared to control); their number was: 18.9×10^6 and 17.6×10^6 JFK /1 g of biofilm (fig. 8, 9). Statistical analysis showed that the differences between the average amounts of copiotrophic bacteria and a kind of filter were statistically significant ($p = 0.00001$ for $\alpha = 0.05$) (fig. 10), and that differences between the average

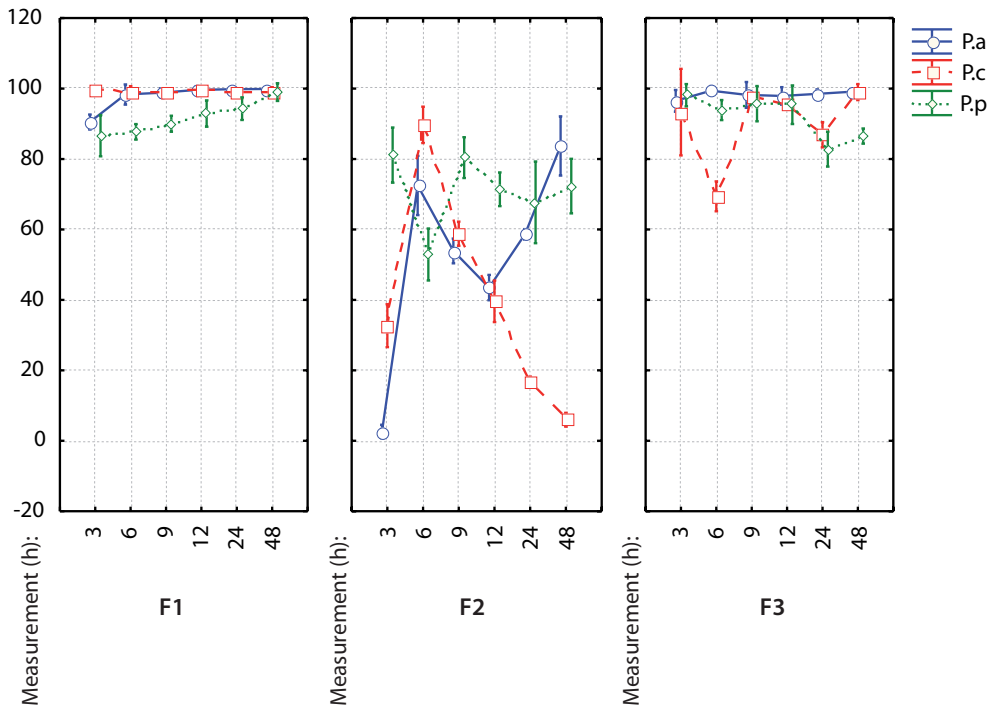

Figure 7. The reduction of pathogens *P. alni* (Pa), *P. cactorum* (Pc) and *P. plurivora* (Pp) at the time of from 3 to 48 hours for three of the filters F1 (control) F2 (phosphite) and F3 (PCNB).

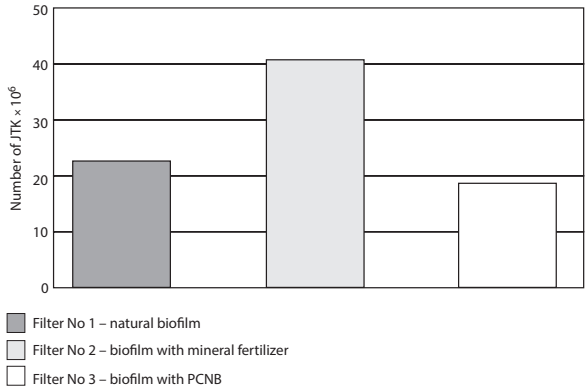

Figure 8. The number of copiotrophic bacteria in biofilm samples taken from three variants of SSF filters working in parallel in the IBL greenhouse (n = 3)

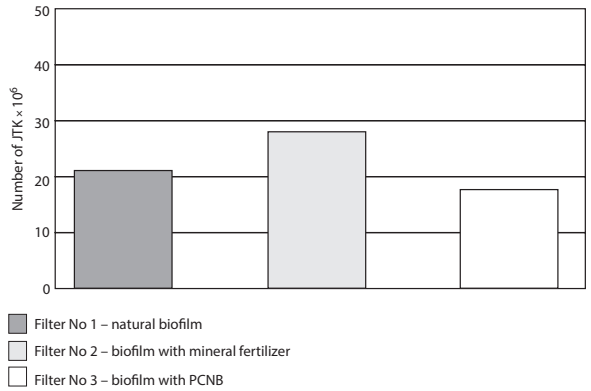

Figure 9. The number of oligotrophic bacteria in biofilm samples taken from the three variants of SSF filters working in parallel in the Forest Research Institute (IBL) greenhouse (n = 3)

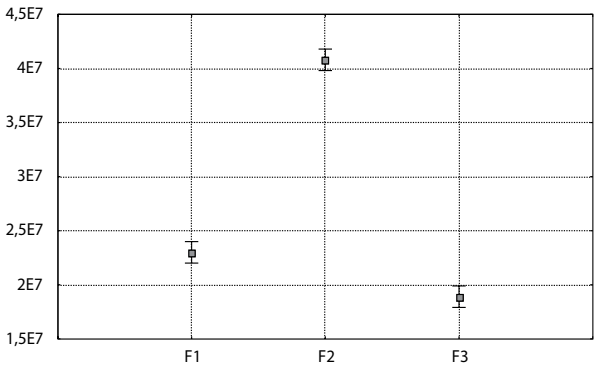

Figure 10. Measurement of the average number of copiotrofic bacteria (copio) +/- in three variants of filters: F1 (control), F2 (phosphite), and F3 (PCNB).

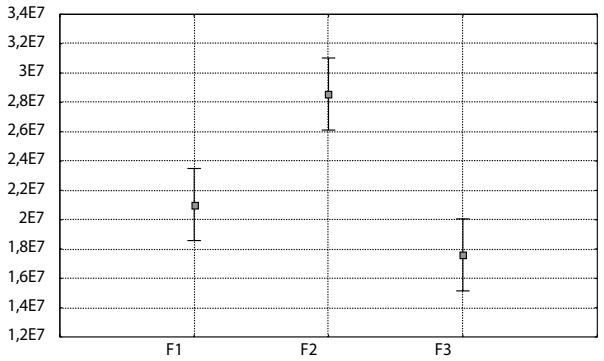

Figure 11. Measurement of the amount of oligotrophic (oligo) bacteria +/- in three variants of filters: F1 (control), F2 (phosphite), and F3 (PCNB).

amounts of oligotrophic bacteria and the type of filter were statistically significant ($p = 0.04867$ for $\alpha = 0.05$) (fig. 11). In the case of oligotrophic bacteria p-values are on the border of significance (at $p = 0.05$).

4. Summary

Literature focuses on the construction, operation, and effectiveness of the SSF type of sand filters in removing phytopathogens from the contaminated water. Not much research describes modifications of biofilms by the addition to filtered water substances such as fungicides or fertilizers. In our experiments, pollution of filtered water originated from agriculture reduced the performance of SSF biofilters, interfering their efficiency of the elimination of phytopathogens. Both the mineral fertilizer as well as PCNB influenced the SSF biofilm microbial balance. The quantitative composition and species of bacteria and microscopic fungi was changed, which is also confirmed in the literature (Macedo et al. 2007; Barragán-Huerta et al. 2007; Davey, O'toole 2000). It was observed that the presence of a mineral fertilizer in the filtered water decreased the elimination of phytopathogens more than the performance of SSF filtration of water containing fungicide, PCNB. Biofilm's efficiency depends on the spatial structure and species' composition of the microbial communities that inhabit them (Davey, O'toole 2000). Both of these items, unless they are violated, ensure the proper functioning of the alive and dead elements of biofilm as a whole, which is consistent with the results of other authors (Lewandowski et al. 1993; Moller et al. 1996). In water and soil environment under the influence of mineral –

nutrients – fertilizers both the quantity and species of microorganisms are in the realignment form that mainly starts growing microorganisms with high nutritional requirements, which are widespread in the environment (Koch 2001). They are biologically less active than microorganisms with medium or very poor nutritional requirements (Kuznetsov et al. 1979; Alabouvette 1986). It was found that the presence of fungicide (PCNB) in filtered water probably slowed down the development of microscopic fungi normally inhabiting the SSF biofilter. Microscopic fungi in the community of microbial biofilms play a dual role, because they produce exogenous active substances (enzymes) and due to the complex structure of the mycelia are additional mechanical barrier for pollutants carried with water flowing through the biofilter (Wohanka 1995). Integrated protection of nurseries has been validated legally by orders of the European Commission (EU) (Directive No. 2009/128/EC of 21.10.2009 and Regulation No 546/2011 of 10.06.2011 on the integrated protection of plants against pests). This idea is based on the complementary use of several (or all) of the possible methods for plant protection. Legislatives were established to protect the environment in order to reduce the proportion of chemicals (pesticides), which residues are found in drinking water (Aslan 2005). Accordingly, the Ministry of Agriculture and Rural Development elaborated a project of the national action plan to reduce the risks associated with the use of plant protection products during 2013-2017. The tests performed have shown the ability to remove fertilizer from water using SSF at the expense of losing efficiency in eliminating filter phytopathogens. This phenomenon was not observed (not statistically significant) in the presence of fungicide, PCNB, probably because it does not affect so strongly the bacteria present in the biofilm than the fertilizer added to the filtered water. In the presence of water pollution of agricultural origin one must take into account the deterioration of the filtering properties of biofilms. Common presence of phytopathogens in surface waters is crucial for the health of seedlings, if water is used for watering the plants for plantings. Without filtering, pathogens will be easily distributed in nurseries. It should be noted that the increased number of sand filters (working under pressure) are not as effective as biological filters (SSF) due to too rapid flow of water through a bed of sand. They can only capture the weed seeds. The offered new technologies like the use of membrane filters or application of UV radiation are effective but very expensive. In contrast, the use of such chemicals like sodium hypochlorite is cheaper but contrary to the principles of environmental protection (Wohanka 1995). Water resources in Poland per capita amounts to one-third of the European average, so in the future, the water drawn from deep wells will be a privilege (taxed accordingly), or developing international environmental legislation generally will prohibit its use for this purpose, hence the need to increase the use of natural water surface, although they contain a lot of human and plant pathogens (pathogenic bacteria). Nurseries with appropriate filter systems for this task will be better prepared and will be more competitive on the market of the nursery plants production. Sand filters of SSF type will become particularly important in the nursery, arising in the future nursery farm with a closed water circulation, perhaps with a set of filters in sequence to remove residues of pesticides and fertilizers, and phytopathogens. Closed water circuits installed in nurseries contribute significantly to water economy. Perhaps, the Polish State Forests will follow German nurseries soon and will build the first full-size type SSF filter in one of the nurseries in Poland. Interested forest districts could then benefit from its experience and become convinced about the efficacy of water treatment as a part of integrated plant protection in nursery practice. This will limit the use of pesticides to a minimum and thus minimize the pressures on the environment and will have a beneficial effect on the conservation of forest biodiversity.

5. Conclusions

1. Natural slow sand filters are effective in removing pathogens of the genus *Phytophthora*, only when water is contaminant-free from agricultural sources (fertilizers, except fungicide PCNB).

2. It is not recommended to add the nitrogen-containing fertilizer and phosphites to tanks storing water for watering plants in nurseries, as it reduces the effectiveness of the biofilm.

3. If adding of fertilizer to the water reservoir for irrigation is necessary, then water intended for watering the plants should be filtered for at least 48 hours.

4. Addition of a mineral fertilizer (containing nitrogen and phosphites) and fungicide (PCNB) causes changes in the size and the composition of bacteria colonizing the biofilm, which affects the efficiency of the elimination of pathogens by the SSF.

5. Adding of fertilizer increases over double the number of bacteria in the biofilm, both copiotrophic and oligotrophic, as compared to the control.

6. Addition of PCNB reduces the number of bacteria, both copiotrophic and oligotrophic by nearly one-fifth compared to the control.

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Source materials:

Dyrektywa Parlamentu Europejskiego i Rady 2009/128/WE z dnia 21 października 2009 r. ustanawiająca ramy wspólnotowego działania na rzecz zrównoważonego stosowania pestycydów.

Rozporządzenie Komisji (UE) NR 546/2011 z dnia 10 czerwca 2011 r. wykonujące rozporządzenie (WE) nr 1107/2009 Parlamentu Europejskiego i Rady w odniesieniu do jednolitych zasad oceny i udzielania zezwolenia na środki ochrony roślin.